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THE TWO ISOFORMS OF RAT METALLOTHIONEIN ARE COORDINATELY REGULATED IN VIVO

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

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B.S., Weber State University, 1994

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Diane M. Todd ENTITLED The Two Isoforms of Rat Metallothionein are Coordinately Regulated In Vivo BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Todd, Diane M. M.S., Department of Pharmacology/Toxicology, Wright State University, 2003. The Two Isoforms of Rat Metallothionein are Coordinately Regulated In Vivo.

Metallothionein (MT) is an inducible protein whose unique structure contributes to its many functions. One of MT's functions is the detoxification of heavy metals. Cadmium (Cd), which is an environmental pollutant and a hazard to both humans and animals, is detoxified by MT. There are four known MT isoforms (MT I-IV) and this study focuses on only MT I and MT II. Even though a lot is known about the effect of Cd on MT induction little has been reported about the MT isoforms and their pattern of mRNA expression. It is the aim of this study to investigate the hypothesis that the two isoforms of rat metallothionein are coordinately regulated in vivo. To test this hypothesis, rats were dosed intraveneously via the lateral tail vein with 0.0, 0.5, 1.0 or 2.0 mg/kg Cd acetate and the mRNA levels in the liver tissue were determined by Real-Time PCR at various time points (0, 0.5, 1, 3, 6, 9, 12, 18 and 24 hours). The fold increase in mRNA expression was determined using the comparative C_T method and plotted versus time. MT I mRNA exhibits a similar induction pattern at the 0.5, 1.0 and 2.0 mg/kg doses up to the 6 hour time point. By 12 hours both the 0.5 mg/kg and 1.0 mg/kg doses decline to baseline levels whereas the 2.0 mg/kg dose remains elevated. The pattern of induction for MT II mRNA is similar for all three doses up to the 9 hour time point. At

the 0.5 mg/kg and 1.0 mg/kg doses MT II mRNA declines to baseline at the 12 hour time point and then increases at 24 hours. At the 2.0 mg/kg dose, MT II mRNA remains elevated at times greater than 12 hours. In comparing MT I and MT II mRNA expression, the results demonstrate that at all doses, MT II peaks first followed by a decline at 3-6 hours at which time MT I peaks. MT I mRNA decreases between 6 and 12 hours, while MT II mRNA peaks at 9 hours. At the 0.5 mg/kg and 1.0 mg/kg dose both MT I and MT II decrease at the 12 hour time point and then increase at 24 hours while at the 2.0 mg/kg dose, both mRNAs remain elevated between 12 and 24 hours. By comparing MT I and MT II mRNA patterns of induction it is evident that the two isoforms are not coordinately regulated in vivo.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABBREVIATIONS

As	arsenic
Bi	bismuth
β-me	beta-mercaptoethanol
Cd	cadmium
Cd-MT	cadmium-metallothionein
Cd-P	cadmium-protein
cDNA	complementary deoxyribose nucleic acid
C_T	threshold cycle
Cu	copper
Cys	cysteinyl
ΔC_T	delta threshold cycle
$\Delta\Delta C_T$	delta-delta threshold cycle
DEPC	diethyl pyrocarbonate
DNA	deoxyribose nucleic acid
DTT	dithiothreitol
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Hg	mercury
MANOVA	multivariate analysis of variance
MRE	metal responsive element
MTF-1	metal transcription factor-1
mRNA	messenger ribose nucleic acid

MT	metallothionein
NAC	no amplification control
NTC	no template control
P	protein
Pb	lead
PCR	polymerase chain reaction
RPM	revolutions per minute
rRNA	ribosomal ribose nucleic acid
t½	half life
TE	tris-EDTA
T_m	melting temperature
UNG	uracil n-glycoslyase
UNK	unknown
Zn	zinc
Zn-MT	zinc-metallothionein
Zn-IN	zinc-sensitive inhibitor
Zn-P	zinc-protein
ZnT-1	zinc-transporter protein

INTRODUCTION

Cadmium Toxicity

Cadmium (Cd) ranks close to lead (Pb), arsenic (As) and mercury (Hg) in toxicological importance due to increasing levels in the environment (Friberg et al., 1986). Cd is a ubiquitous environmental contaminant that is classified as carcinogenic to humans and animals (IARC, 1993). A major source of Cd ingestion in animals and humans has been due to food contamination, namely from grains and shellfish. Also industrial activity can significantly increase the distribution of Cd in the environment. Occupational exposure usually occurs from dust and aerosols that are typically produced during the smelting and refining of metal ores, electroplating or welding and during the manufacture of pigments, plastic stabilizers, and nickel-cadmium batteries. causing pulmonary carcinogenesis (IARC, 1993). Cigarette smoke and contaminated food, water and air represent other possible sources of Cd exposure (Elinder, 1986). Acute exposure to Cd has been associated with damage to the testes, lungs and liver, while chronic exposure can lead to emphysema, obstructive airway disease, permanent renal dysfunction, bone disorders, and immunotoxicity (Friberg et al., 1986).

After an acute exposure, the liver accumulates 60% of administered Cd (Frazier and Puglese, 1978; Cain and Skilleter, 1980) and the liver is the main target of Cd toxicity (Meek, 1959; Dudley et al., 1982). A hepatotoxic dose of Cd will cause parenchymal cell swelling and cytoplasmic eosinophilia within 1 hour after injection, while severe necrosis will occur 10-20 hours later (Dudley et al., 1982, 1984), and death due to liver failure can

occur within 24 hours (Hoffmann et al., 1975; Dudley et al., 1982, 1984; Goering and Klassen, 1983).

Exposure of biological organisms to toxic chemicals in the environment has been a common occurrence over time. Due to these exposures, organisms from bacteria to human beings have developed defense mechanisms to handle toxic elements such as Cd. One defense mechanism involves induction of unique detoxifying proteins. MT is one such inducible protein that is involved in the detoxification of heavy metals in general and cadmium in particular.

Metallothionein

MT was first discovered in 1957 in horse kidney (Margoshes and Vallee, 1957). MT is present in many other organs such as the liver, intestine, pancreas and brain. Synthesis of hepatic MT is induced by a number of metals, cytokines, and stress hormones as well as by a wide range of chemicals that act indirectly via a stress or inflammatory response (Hamer, 1986; Bremner, 1987). The absence of MT I and MT II has been shown to increase inorganic Cd-induced lethality and hepatotoxicity, whereas overexpression is associated with protection (Klaassen and Liu, 1998). Numerous studies have shown the ability of Cd to displace Zn from MT and to induce the synthesis of MT (Kagi, 1993).

Structure

Mammalian MTs are single-chained polypeptides consisting of 61 to 68 amino acid residues. They are low molecular weight proteins (6-7 kDa) that are cysteine-rich and contain a high metal content. Even though as many as 18 different metals may associate

with MT, the metal content of endogenous MTs consists of primarily zinc (Zn), copper (Cu) or Cd. There are 18-23 cysteine residues in MT with no aromatic amino acids or histidines. The cysteine residues are highly conserved and form a Cys-X-Cys distribution, where X stands for an amino acid residue other than Cys. Metals bind to MT as metal-thiolate (mercaptid) complexes and form regional clusters within the tertiary protein structure (Hamer, 1986). MT contains two functional domains: the more stable α-domain (C-terminal) and the more reactive β-domain (N-terminal). Normally there are seven atoms of Zn bound to MT. Four atoms are bound in the α-domain and three atoms are bound in the β-domain. It is these unique structural characteristics that endow MT with potent metal-binding and redox capabilities (Figure 1).

Function

MTs are found in nearly all-living organisms and in most tissues. The liver, kidney, intestine and pancreas have been shown to contain the highest concentration of mammalian MT in the body (Cousins, 1985; Bremner and Beattie, 1990; Nath et al., 1988; Hamer, 1986). MT is primarily found in the cytosol of cells but immunohistochemical studies have shown increased MT expression in both the cytoplasm and nucleus of rapidly proliferating cells (Miles et al., 2000; Davis and Cousins, 2000). The exact function of nuclear MT is unknown but it has been postulated that it may protect DNA from oxidative damage or regulate the supply of Zn to crucial enzymes and transcription factors necessary for cell division (Moffatt and Denizeau, 1997; Cherian and Apostolova, 2000; Ogra and Suzuki, 2000).

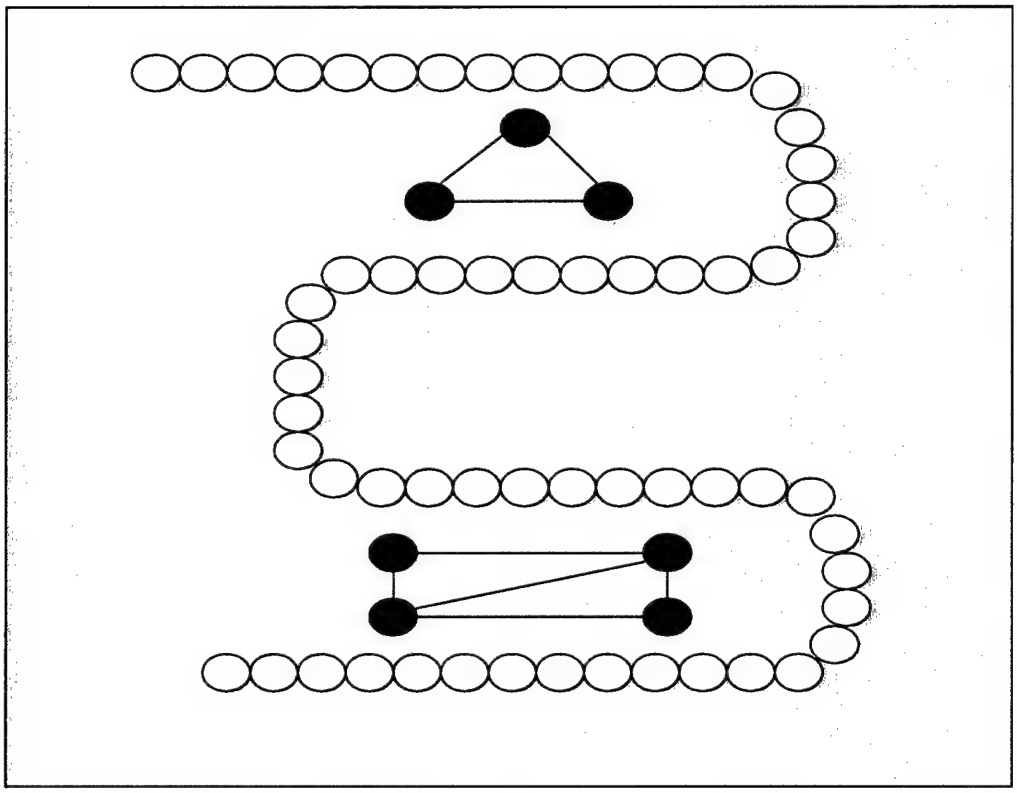

Figure 1. Structure of MT. The white circles within the chain represent the amino acids and the yellow circles represent the cysteine residues. The triangle represents the beta domain with 3 atoms of Zn (blue circles) bound while the rectangle represents the alpha domain with 4 atoms of Zn bound.

MT has many functions and is involved in the detoxification of toxic metals (Cd and Hg), homeostasis of essential metals (Zn and Cu), and serves a protective role against oxidative damage caused by free radicals. In Cd toxicity, MT plays a protecting rolenot just against hepatotoxicity, but also nephrotoxicity, hematotoxicity, immunotoxicity and bone damage (Klassen and Choudhuri, 2000; Nordberg et al., 2000). MT plays an important role in Cd detoxification (Durnam and Palmiter, 1987) while protecting against Cd toxicity (Goering and Klaassen, 1983, 1984).

Metallothionein Isoforms

All vertebrates contain two or more distinct MT isoforms designated MT I through MT IV (Moffatt and Denizeau, 1997). In mammals, MT I and MT II are present in all tissues (Searle et al., 1984; Hamer, 1986) whereas MT III is expressed mainly in the brain (Palmiter et al., 1992) and MT IV is most abundant in certain stratified squamous epithelium (Quaife et al., 1994). Not much is currently known about the function of MT IV. Different genes encode each of these isoforms. The expression of some of these genes is under separate control and may serve different biological purposes (Sadhu and Gedamu, 1988; Jahroudi, et al. 1990). The coding and translated amino acid sequences for MT I and MT II exhibit the differences in amino acid composition between the two isoforms. (Figures 2 & 3).

A hallmark of the MT I and MT II genes is their transcriptional induction by Zn and Cd (Andrews 1990). These metals are the common inducer of MT genes and all MT promoters contain multiple copies of a semiconserved sequence that is reponsible for induction by metals, called metal-responsive elements (MRE) (Culotta and Hamer 1989).

MT I Coding & Translated Amino Acid Sequences

```
                10          20          30          40          50
5' ATG GAC CCC AAC TGC TCC TGC TCC ACC GGC GGC TCC TGC ACC TGC TCC AGC 3'
   TAC CTG GGG TTG ACG AGG ACG AGG TGG CCG CCG AGG ACG TGG ACG AGG TCG
   Met Asp Pro Asn Cys Ser Cys Ser Thr Gly Gly Ser Cys Thr Cys Ser Ser

            60          70          80          90          100
5' TCC TGC GGC TGC AAG AAC TGC AAA TGC ACC TCC TGC AAG AAG AGC TGC TGC 3'
   AGG ACG CCG ACG TTC TTG ACG TTT ACG TGG AGG ACG TTC TTC TCG ACG ACG
   Ser Cys Gly Cys Lys Asn Cys Lys Cys Thr Ser Cys Lys Lys Ser Cys Cys

            110         120         130         140         150
5' TCC TGC TGC CCC GTG GGC TGC TCC AAA TGT GCC CAG GGC TGT GTC TGC AAA 3'
   AGG ACG ACG GGG CAC CCG ACG AGG TTT ACA CGG GTC CCG ACA CAG ACG TTT
   Ser Cys Cys Pro Val Gly Cys Ser Lys Cys Ala Gln Gly Cys Val Cys Lys

          160         170         180         190         200
5' GGT GCC TCG GAC AAG TGC ACG TGC TGT GCC TGA AGT GAC GAA CAG TGC TGC 3'
   CCA CGG AGC CTG TTC ACG TGC ACG ACA CGG ACT TCA CTG CTT GTC ACG ACG
   Gly Ala Ser Asp Lys Cys Thr Cys Cys Ala Ter

        210         220         230         240         250
5' TGC CCT CAG GTG TAA ATA ATT TCC GGA CCA ACT CAG AGT CTT GCC GTA CAC 3'
   ACG GGA GTC CAC ATT TAT TAA AGG CCT GGT TGA GTC TCA GAA CGG CAT GTG

      260         270         280         290         300
5' CTC CAC CCA GTT TAC TAA ACC CCG TTT TCT ACC GAG CAT GTG AAT AAT AAA 3'
   GAG GTG GGT CAA ATG ATT TGG GGC AAA AGA TGG CTC GTA CAC TTA TTA TTT

      310
5' AGC CTG T 3'
   TCG GAC A
```

Figure 2. MT I coding and translated amino acid sequences. The letters highlighted in red represent the MT I forward and reverse primers. The bold letters are the corresponding amino acids.

MT II Coding & Translated Amino Acid Sequences

```
                10          20          30          40          50
5' ATG GAC CCC AAC TGC TCC TGT GCC ACA GAT GGA TCC TGC TCC TGC GCT GGC 3'
   TAC CTG GGG TTG ACG AGG ACA CGG TGT CTA CCT AGG ACG AGG ACG CGA CCG
   Met Asp Pro Asn Cys Ser Cys Ala Thr Asp Gly Ser Cys Ser Cys Ala Gly

            60          70          80          90          100
5' TCC TGC AAA TGC AAA CAA TGC AAA TGC ACC TCC TGC AAG AAA AGC TGC TGT 3'
   AGG ACG TTT ACG TTT GTT ACG TTT ACG TGG AGG ACG TTC TTT TCG ACG ACA
   Ser Cys Lys Cys Lys Gln Cys Lys Cys Thr Ser Cys Lys Lys Ser Cys Cys

           110         120         130         140         150
5' TCC TGC TGC CCC GTG GGC TGT GCG AAG TGC TCC CAG GGC TGC ATC TGC AAA 3'
   AGG ACG ACG GGG CAC CCG ACA CGC TTC ACG AGG GTC CCG ACG TAG ACG TTT
   Ser Cys Cys Pro Val Gly Cys Ala Lys Cys Ser Gln Gly Cys Ile Cys Lys

          160         170         180         190         200
5' GAG GCT TCG GAC AAG TGC AGC TGC TGC GCC TGA AGT GGG GGC GTC CTC ACA 3'
   CTC CGA AGC CTG TTC ACG TCG ACG ACG CGG ACT TCA CCC CCG CAG GAG TGT
   Glu Ala Ser Asp Lys Cys Ser Cys Cys Ala Ter

        210         220         230         240         250
5' ATG GTG TAA ATA AAA CAA CGT AAG GAA CCT AGC CTT TTT TTG TAC AAC CCT 3'
   TAC CAC ATT TAT TTT GTT GCA TTC CTT GGA TCG GAA AAA AAC ATG TTG GGA

      260         270         280         290         300
5' GAC CGG TTC TCC ACA CTT TTT TCT ATA AAG CAT GTA ACT GAC AAT AAA ATA 3'
   CTG GCC AAG AGG TGT GAA AAA AGA TAT TTC GTA CAT TGA CTG TTA TTT TAT

    310
5' AAA AAA C 3'
   TTT TTT G
```

Figure 3. MT II coding and translated amino acid sequences. The letters highlighted in red represent the MT II forward and reverse primers. The bold letters represent the corresponding amino acids.

MREs are critical for the induction of MT genes and have been shown to confer response to Zn and Cd (Stuart et al. 1984; Stuart et al. 1985). They contain a 12- to 15-base pair sequence consisting of a highly conserved heptanucleotide core, TGC (A/G)CNC, and a less conserved flanking sequence (Kim et al. 1993). MREs are present in multiple copies in the MT promoter region, and they appear to be variable in their response to metal-induced transcription. They have been shown to interact with a variety of nuclear proteins that either activate or inhibit transcription (Miles et al. 2000; Tang et al. 1999; Ogra et al. 2001).

Metal transcription factor (MTF-1) is a protein responsible for transactivation of MT genes through the MRE. In humans, only four out of seven MREs react with MTF-1 to mediate a Zn response (Koizumi et al., 1999). Even though, Zn, Cd and Bi metals will activate the promoter of the MT-gene via MREs (Palmiter 1994), only Zn is specific for binding and activating MTF-1 (Andrews 2000). The binding of Zn to MTF-1 allows the protein to bind to MREs in the promoter region, which in turn initiates MT-gene transcription (Figure 4). It has been proposed that MTF-1 regulates the free Zn concentration by controlling the expression of MT as well as that of a Zn-transporter protein, ZnT-1 (Langmade et al. 2000). A Zn-sensitive inhibitor that prevents MTF-1 from binding to MREs may control the basal expression of MTF-1. Elevation of the intracellular Zn concentration dissociates the inhibitor from MTF-1, thereby promoting transcription of MT (Palmiter 1994). Zn has also been shown to prolong the nuclear retention of MTF-1, but whether this further promotes transcription is unclear (Otsuka et

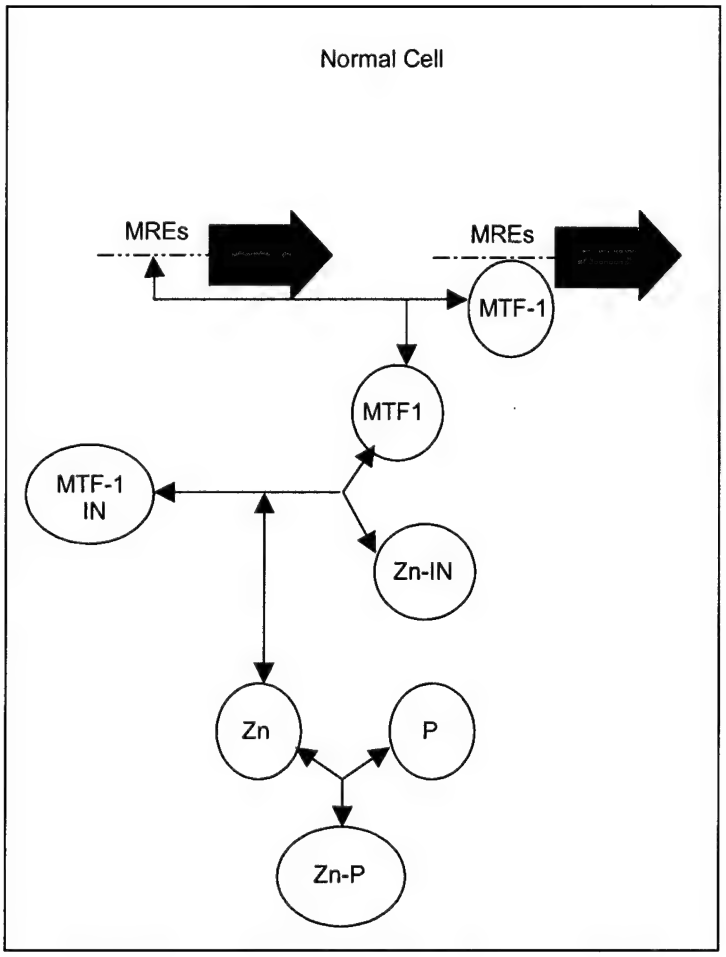

Figure 4. Proposed mechanism of MT gene regulation under normal cellular conditions. Under normal cellular conditions Zn is bound to cellular proteins within the cell (Zn-P) and MTF-1 is normally bound to the Zn-sensitive inhibitor (IN). Transcription is occurring but at a minimal rate. The size of the circle relates to the amount present within the cell.

al. 2000). MTF-1 is important in the regulation of a number of genes that play a role in cellular response to various stresses (Lichtlen et al. 2001).

MT I and MT II are also induced by a variety of stress conditions and compounds, including glucocorticoids, cytokines, reactive oxygen species and metal ions (Kagi, 1991). MT III and MT IV are unresponsive to these inducers. Metal ions such as Cd and Zn are the most potent inducers of MT I and MT II. The high metal-inducibility of MT I and MT II has been linked with their role in heavy-metal detoxification (Moffatt and Denizeau, 1997).

Hypothesis

Both MT I and MT II genes have been shown to be coordinately regulated in the mouse and their protein products are thought to be functionally equivalent (Searle et al., 1984; Kagi, 1993). However, discrepancies between tissue levels of MT mRNA and MT protein have been widely reported (Andersen et al., 1983; Lehman-McKeeman et al., 1988; Paynter et al., 1990; Iijima et al., 1990; McCormick et al., 1991; Misra et al., 1997; Carginal et al., 1998). Unfortunately, in some cases, the individual MT isoforms and/or their associated mRNAs were not examined.

The hypothesis was proposed that the two isoforms of MT are coordinately regulated in vivo at the mRNA level. To test this hypothesis the mRNA level of both MT I and MT II were evaluated in the liver of F344 male rats following a single intravenous dose of 0, 0.5, 1.0 or 2.0 mg/kg Cd acetate in the lateral tail vein. Time series data was evaluated at 0, 0.5, 1, 3, 6, 9, 12, 18 and 24 hours post dose. Groups of four animals were sacrificed at each specified time point and the livers collected and flash frozen in liquid nitrogen. MT I and MT II mRNA levels were determined by Real-Time PCR. These

time series data were used to compare the effects of Cd on the induction of both MT I and MT II mRNA levels. The results suggest that the hypothesis should be rejected, i.e. the data do not support the hypothesis that the two isoforms of MT are coordinately regulated at the mRNA level.

METHODS AND MATERIALS

Animals and Treatment

Male Fisher (F344) rats (230-275g) were obtained from Charles River Laboratories, Raleigh, NC. All animals used in this study were handled in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1996, as amended.

Rats were housed individually with each lot segregated from all other rodents for a 7-10 day quarantine/acclimation period. All animals were observed at least twice daily for general health status and any signs of illness were recorded. A polycarbonate shoebox caging system with cellulose fiber contact bedding (Cell-Sorb Plus, A.W. Products, Inc., New Philadelphia, OH) was the primary housing unit. Rats were changed into freshly bedded and sanitized cages at least once per week. Rodent chow (#5008, Purina Mills, Inc., St. Louis, MO) and fresh conditioned (reverse osmosis) water was available *ad libitum*. Prior to surgery all rats were kept in sanitized animal holding rooms designed to provide 10-15 complete fresh air changes per hour. Room air temperature and humidity were maintained between 21-26°C and 30-70%. Rodent cage racks remained inside Bio-Clean mass air displacement units, which provided a constant supply of HEPA filtered air. Electronically controlled full spectrum fluorescent light was provided on a 12:12 hour light:dark cycle.

Rats were housed in individual metabolism cages for 3 days prior to dosing to acclimate and collect control urine. Each rat was dosed intravenously in the lateral tail vein with 0.5, 1.0 or 2.0 mg/kg Cd, prepared with cadmium acetate in sterile physiological saline. At 0, 0.5, 1, 3, 6, 9, 12, 18 and 24 hours post dose, groups of 4 animals were sacrificed by CO_2 inhalation. Blood was drawn via the inferior vena cava, and liver, kidney, fat, muscle, lung, brain, skin and femur bone were collected. Livers were perfused with 0.1 M Tris-acetate buffer (pH 7.4), and a small lobe was removed and fixed in 10% buffered formalin. The remaining liver and all other tissues were flash frozen in liquid nitrogen and stored at -80°C until processing.

Sham rats were intravenously dosed in the lateral tail vein with Cd-free saline. Groups of 4 rats were then sacrificed by CO_2 inhalation at 1, 5, 9, 13, 17 and 21 hours after dosing. Blood and tissues were removed in the same manner as those from the Cd-treated animals. All liver tissue was stored at -80°C prior to analysis.

RNA Isolation

Total RNA was isolated from rat livers using the RNeasy® Mini Kit (Qiagen Inc., Valencia, CA). The *RNeasy Mini Protocol for Isolation of Total RNA from Animal Tissues* was followed with minor modifications. RNA lysis Buffer RLT was prepared by adding 10 µl β-mercaptoethanol (β-ME) per 1 ml of RNeasy Lysis Buffer (Buffer RLT) in a fume hood. Approximately 30 mg of liver tissue was added to 600 µl of RNA lysis Buffer RLT in a 2 ml collection tube. A stainless steal bead was added to each 2 ml tube containing the RNA lysis Buffer RLT and liver tissue. The tissue was homogenized on the Mixer Mill MM 300 (F. Kurt Retsch GmgH & Co. KG, Haan, Germany) for 2

minutes at 20 Hz. The Mixer Mill rack was rotated to allow even homogenization and homogenized for another 2 minutes at 20 Hz. The tissue sample (including the bead) was centrifuged for 3 minutes at 15,000 rpm in a Micromax microcentrifuge (International Equipment Co., Needham Heights, MA). The supernatant was carefully transferred to a 2ml collection tube and 600 µl of 70% ethanol was added. Each tube was mixed well by pipetting. To a RNeasy mini column placed in a 2 ml collection tube, 700 µl of sample was added. The tube was gently closed and centrifuged for 25 seconds at 15,000 rpm. The flow-through was decanted and the column blotted. This was repeated until the entire sample was processed. To wash the column, 700 µl of Buffer RW1 was applied to the column and the column was centrifuged for 25 seconds at 15,000 rpm. The flow-through was discarded and the Buffer RW1 wash was performed again. After the second Buffer RW1 wash, the flow-through was discarded and the RNeasy column was transferred to a new 2 ml collection tube. Another wash was performed by adding 500 µl Buffer RPE onto the RNeasy column and centrifuging for 25 seconds at 15,000 rpm. The flow-through was discarded and another 500 µl Buffer RPE was added to the column, it was centrifuged 2.15 minutes at 15,000 rpm, and the flow-through discarded. To dry the column and prevent any chance of Buffer RPE carryover, the column was centrifuged another 1.15 minutes at 15,000 rpm. To elute the RNA, the RNeasy column was transferred to a new 1.5 ml collection tube and 30 ul RNase-free water was pipetted directly onto the RNeasy silicia-gel membrane. The RNeasy column was allowed to sit at room temperature for 1 minute and centrifuged for 1.15 minutes at 15,000 rpm. This step was performed twice and the eluates were combined and placed on ice. A 1:50 dilution was made in a 1.5 ml tube by adding 2 µl total RNA extract to 98 µl 1X TE. The

sample was vortexed and the concentration of total RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a GeneQuant Pro spectrophotometer (Biochrom Ltd., Cambridge England). The purity of the total RNA was determined by the ratio (A_{260}/A_{280}). A ratio of 1.9-2.1 indicated a pure RNA sample. The integrity of the RNA was determined after separation by agarose gel electrophoresis by illuminating the 28S and 18S ribosomal RNA bands with ethidium bromide (Figure 5). The bands were viewed by UV light using the Fisher Biotech Electrophoresis System Transilluminator (Fisher Scientific, Pittsburgh, PA).

The RNA samples were aliquoted into 5 μg aliquots and stored in the -80°C freezer until processed to cDNA.

First Strand Synthesis of cDNA by Reverse Transcription

First strand synthesis of cDNA from total RNA was performed using the 5 μg total RNA aliquots. All of the reagents in this procedure were thawed on ice and quick-spun (except the enzyme). To each 5 μg sample, 1 μl 100 pmol/ul oligo dt-T7 promoter and the appropriate volume of DEPC water was added to obtain a final reaction volume of 25 μl. Each sample was vortexed (Genie 2 Fisher Scientific, Bohemia, NY) and quick-spun (Stratagene Profuge™ 10K, USA). The samples were then incubated for 10 minutes at 70°C in a GeneAmp® 9700 PCR System (Applied Biosystems, Foster, CA). During the 10 minute incubation the appropriate amount of master mix was prepared by combining 5 μl 5X 1st strand buffer (Invitrogen, Carlsbad, CA), 2 μl 0.1 M DTT (Invitrogen, Carlsbad, CA), and 1 μl 10 mM dNTP mix (Invitrogen, Carlsbad, CA) for each sample. After the 10 minute 70°C incubation, the samples were immediately put on

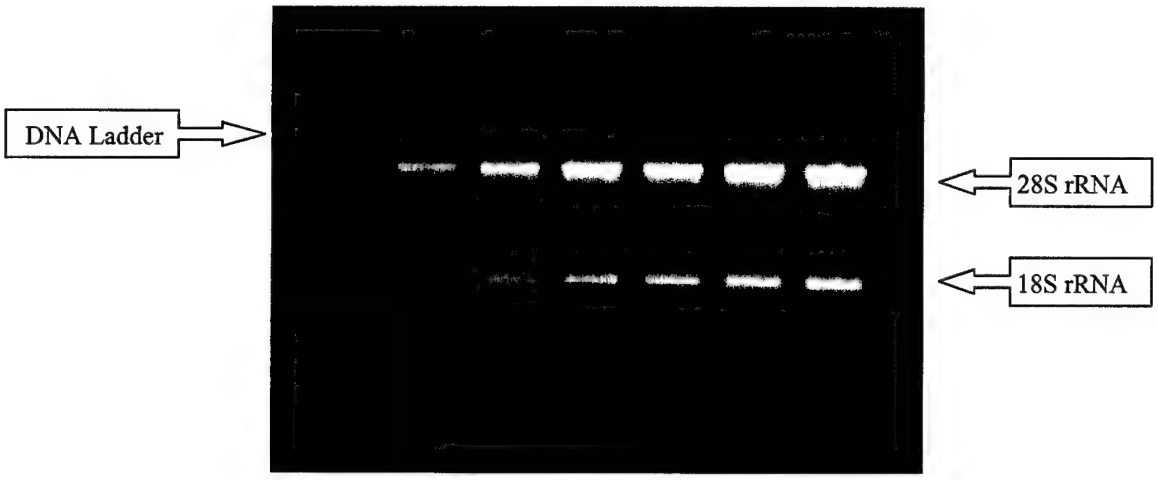

Figure 5. Example of an agarose gel electrophoresis showing 28s and 18s rRNA bands.

ice, vortexed and quick-spun. The samples were incubated for 2 minutes at 42°C in a Model 180 Molecular Biology Water Bath (Precision Scientific, Chicago, IL). After the 2-minute incubation, 8 µl of the master mix was added to each sample. The samples were mixed and incubated another 2 minutes in the 42°C water bath. After the 2-minute incubation, 1 µl Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) was added to each sample and the samples mixed thoroughly. The samples were incubated for 1.5 hours at 42°C in the Gene Amp® 9700 PCR system. Once the incubation was completed the samples were immediately placed on ice, vortexed, quick-spun and stored in the -20°C freezer until used in Real-Time PCR.

Real-Time Polymerase Chain Reaction

Analysis

Real-Time PCR was performed on cDNA samples to quantitate the amount of MT mRNA in Cd treated liver tissue. Primer Express™ software (Applied Biosystems, Foster City, CA) was used to design the primers. The primers used were rat specific MT I (forward primer: 5'-AAT GTG CCC AGG GCT GTG T-3' and reverse primer: 5'-CGT CAC TTC AGG CAC AGC A-3'); rat specific MT II (forward primer: 5'- AAG TGC TCC CAG GGC TGC-3' and reverse primer: 5'-CCC CAC TTC AGG CGC A-3'); and GAPDH (forward primer: 5'-GAT TCT ACC CAC GGC AAG TTC A-3' and reverse primer: 5'-GGT TTC CCA TTG ATG ACC AGC T-3') (Integrated DNA Technologies, Inc., Coralville, IA). All primers were reconstituted in 1X TE buffer to create a stock solution of 1 mM. A 1:20 dilution (5 µl stock solution to 95 µl DEPC) was made to create a working stock concentration of 50 mM.

SYBR® Green PCR Master Mix (SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, dNTPs (with dUTP), Passive Reference 1, and optimized buffer components (proprietary formulation)) (Applied Biosystems, Foster City, CA) and AmpErase® UNG (Uracil N-glycoslyase) was used in all Real-Time PCR reactions. The SYBR® Green I dye's incorporation into a real-time PCR reaction allows the detection of any double-stranded DNA generated during PCR. Target specific probes are not required, however both specific and non-specific products will generate a signal. The incorporation of the hot-start enzyme AmpliTaq Gold® DNA Polymerase minimizes non-specific product formation.

A master mix was made by combining the appropriate volume of SYBR® Green PCR Master Mix, AmpErase® UNG, DEPC water, Forward Primer and Reverse Primer as shown in Table 1. The template (cDNA) was added to each appropriate well.

To each well of a MicroAmp® Optical 96-well Reaction Plate (Applied Biosystems, Foster City, CA), 23 µl of the corresponding master mix (MT I, MT II, GAPDH) was added. All MT I cDNA samples were diluted 1:100 (1µl cDNA to 99 µl TE buffer). To each well, 2 µl of the appropriate template (cDNA) was added to equal a total reaction volume of 25 µl. MicroAmp Optical Caps (Applied Biosystems, Foster City, CA) were applied to the reaction plate. The reaction plate was run on the ABI Prism™ 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using the thermal cycling conditions in Table 2. On the disassociation curve, the melting temperature (T_m) of GAPDH was 80° C, MT I was 83.8° C, and MT II was 83.4° C.

Table 1. Protocol for using SYBR® Green PCR Master Mix.

Reagents	Vol (ul) for one 25 ul rxn	# of Samples	Sample Volume (ul)
2X SYBR Green PCR Master Mix	12.5	106	1325.0
AmpErase UNG (1U/ul)	0.25	106	26.50
Water	8.25	106	874.50
Primer Forward	1	106	106.0
Primer Reverse	1	106	106.0
Template (cDNA)	2	106	212.0
Total volume			2650.0
Total volume incl/Primers & cDNA	25	106	2650.0
Tube 1 (Add MT1 F&R 41 ul)	861		
Tube 2 (Add MT2 F&R 41 ul)	861		
Tube 3 (Add GAPDH F&R 24 ul)	504		
Total volume w/out Primers & cDNA	2226		

Table 2. Real-Time PCR thermal cycle conditions.

Step	AmpErase UNG Incubation (a)	AmpliTaq Gold Activation (b)	PCR		Dissociation Curve Profile		
	HOLD	HOLD	Cycle (40 cycles)		HOLD		
			Denature	Anneal/Extend			
Temp	50° C	95° C	95° C	60° C	95° C	60° C	95° C
Time	2 min	10 min	15 sec	1 min	15 sec	20 sec	15 sec
Volume	25 µl						

a and b = Required for optimal activity of those enzymes

All plates contained no template controls (NTC), no amplification controls (NAC) and unknowns (UNK). All samples were run in triplicate except for the NTC which were run in duplicate. All reaction plates were stored in the -20°C freezer.

Validation Study

Prior to performing Real-Time PCR analysis, a validation experiment was performed to demonstrate that the PCR efficiencies of the target mRNA (MT I and MT II) and endogenous reference mRNA (GAPDH) were approximately equal. A sensitive method for assessing PCR efficiency is to determine how ΔC_T (threshold cycle) varies with template dilution. The absolute value of the slope of the ΔC_T ($C_{T\ (target)} - C_{T\ (reference)}$) versus log RNA input should be less than 0.1.

The total RNA and cDNA samples used in this study were from untreated rat liver tissue. The total RNA isolation and cDNA synthesis was performed as described above. Two-fold serial dilutions (1, 2, 4, 8, 16, 32, 64, and 128) were made using TE buffer and a 5 μg total RNA sample.

Statistical Analysis

The comparative C_T method uses a mathematical formula ($2^{-\Delta\Delta C_T}$) to calculate the amount of target normalized to an endogenous reference and relative to a calibrator. This method was used to determine relative quantitation. C_T values for MT I and MT II amplification were normalized by subtracting the C_T values for GAPDH using the equation ($C_{T\ (MT\ I\ or\ MT\ II)} - C_{T\ (GAPDH)} = \Delta C_T$). The ΔC_T for the control liver (1 hour time point/0 mg/kg dose) was subtracted from the ΔC_T for Cd-treated liver to calculate the

fold change in MT I and MT II expression ($\Delta C_{T\ (Cd\text{-}treated)} - \Delta C_{T\ (control)}$) = $\Delta\Delta C_T$. This represents the fold change in MT I and MT II mRNA expression.

The treatment groups of MT I and MT II mRNA expression were compared by a mulvariate analysis (MANOVA) with a total N of 108. Means were compared using the Tukey comparisons with a Type I error level of $p<0.05$.

RESULTS

Validation Study

Before the treated liver samples could be analyzed by Real-Time PCR, a validation experiment was performed to demonstrate that the PCR efficiencies of the target and reference were approximately equal. The absolute value of the slope should be less than 0.1. Proving that the efficiencies of both the target and reference rates of transcription are approximately equal allowed us to use the comparative C_T method for quantitation. To determine if the two efficiencies were equal, varying concentrations of total RNA were analyzed by Real Time PCR for MT I, MT II and GAPDH using the forward and reverse primers.

The average MT I C_T values were calculated along with the ΔC_T value (Table 3). The log ng of the total RNA was plotted versus the ΔC_T for MT I (Figure 6). The absolute value of the slope of the log ng total RNA versus the ΔC_T was greater than 0.1. Since MT I did not meet the performance criteria, another experiment was performed. In this experiment the serial dilutions of the total RNA were extended to greater dilutions and MT I was reanalyzed (Table 4). From the plot of the ΔC_T versus the log ng total RNA (Figure 7), the slope was -0.0420 which meets the performance criteria. Since MT I required further dilutions to satisfy the performance criteria, all samples that were analyzed for MT I were diluted 1:100.

Table 3. Average C_T Values for MT I and GAPDH

Dilution	ng Total RNA	MT I Average C_T	GAPDH Average C_T	Log ng Total RNA	ΔC_T (MTI-GAPDH)
1	500	15.6±0.2	14.6±0.1	2.7	1.0±0.3
2	250	16.4±0.6	15.2±0.1	2.4	1.2±0.6
4	125	17.2±0.3	16.0±0.2	2.1	1.3±0.4
8	62.5	18.4±0.3	16.7±0.3	1.8	1.7±0.5
16	31.25	19.7±0.4	17.4±1.2	1.5	2.3±1.2
32	15.63	20.7±0.3	18.8±0.6	1.2	1.9±0.7
64	7.81	21.6±0.4	19.8±0.4	0.9	1.9±0.5
128	3.91	23.6±0.3	20.3±1.0	0.6	3.3±1.1
Slope	-0.8693				
Abs Val <0.1	0.8693				

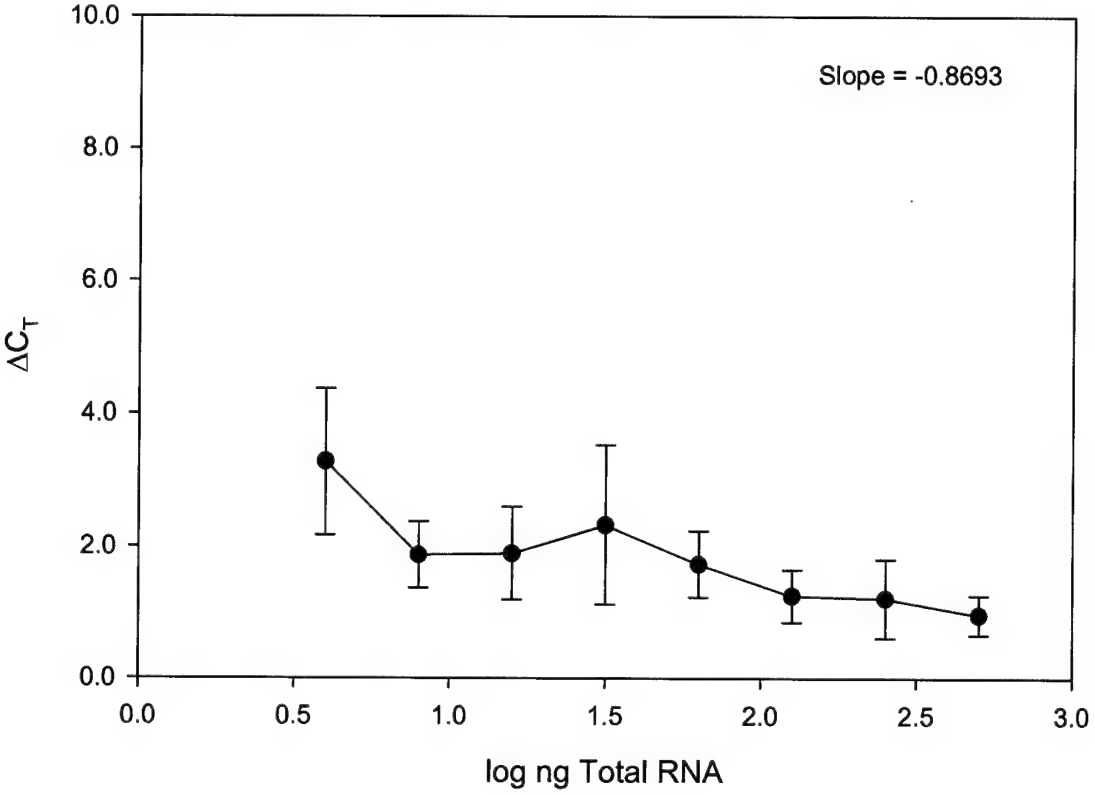

Figure 6. Relative efficiency plot of MT I and GAPDH.

Table 4. Average C_T Values for MT I and GAPDH

Dilution	ng Total RNA	MT I Average Ct	GAPDH Average Ct	Log ng Total RNA	ΔC_T (MTI - GAPDH)
8	62.5	17.0±0.3	15.7±0.1	1.8	1.3±0.4
16	31.25	18.0±0.0	16.5±0.2	1.5	1.5±0.2
32	15.63	18.8±0.2	17.3±0.1	1.2	1.5±0.2
64	7.81	19.9±0.2	18.2±0.2	0.9	1.7±0.3
128	3.91	20.9±0.3	19.2±0.2	0.6	1.7±0.3
256	1.96	21.8±0.5	20.2±0.3	0.3	1.6±0.6
512	0.98	22.6±0.6	21.2±0.1	0.0	1.4±0.6
1024	0.49	23.5±0.3	22.0±0.4	-0.3	1.6±0.4
Slope	-0.0420				
Abs Val <0.1	0.0420				

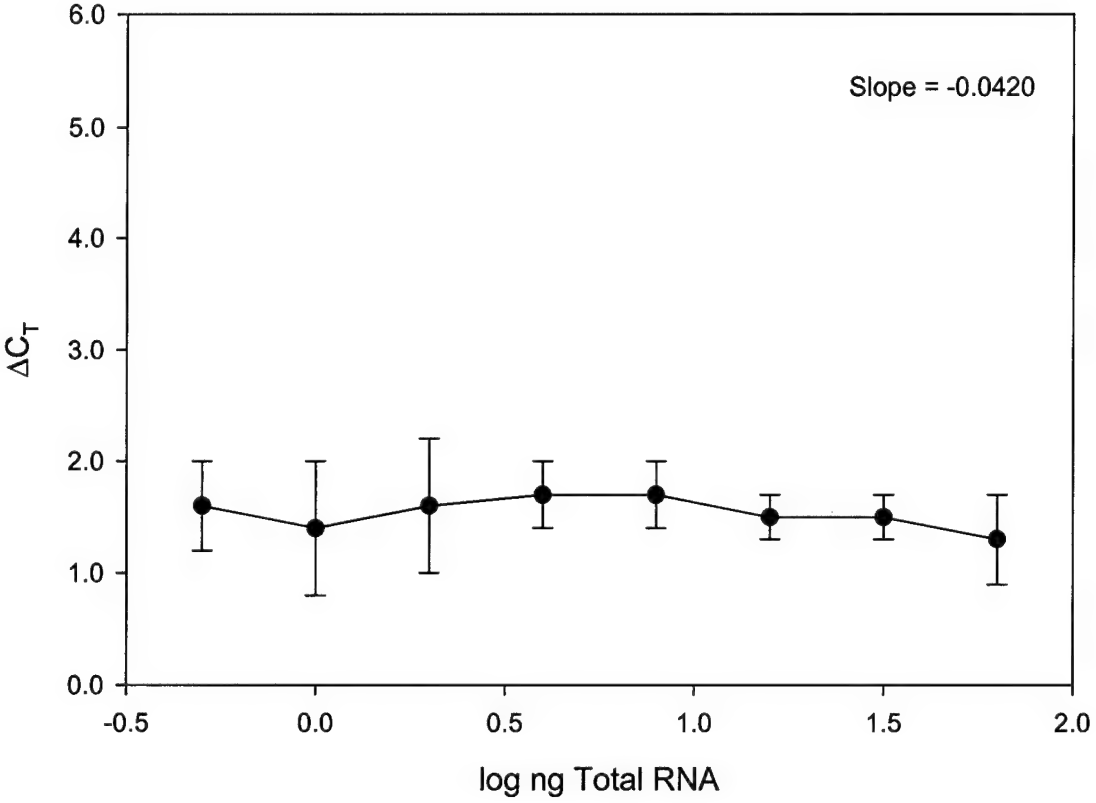

Figure 7. Second relative efficiency plot of MT I and GAPDH.

MT II was analyzed using the same criteria as in the initial experiment for MT I. The average C_T values for MT II and GAPDH were calculated along with the ΔC_T (Table 5). The log ng total RNA was plotted versus the ΔC_T and the slope was determined to be -0.0981 (Figure 8). Since the slope was within the acceptable parameter range, no further validation experiments were performed for MT II.

Effects of Cd on MT I and MT II mRNA Expression

Real-Time PCR was used to determine the time course of mRNA expression of MT I and MT II after exposure to various doses of Cd acetate (0.0 0.5, 1.0 and 2.0 mg/kg) in F344 rats. The time course data for MT I mRNA expression (Figure 9), indicate that the MT I mRNA levels peaked at 6 hours for all three doses. The 2.0 mg/kg dose peaked at the highest level followed by the 1.0-mg/kg dose and the 0.5 mg/kg dose. Both the 0.5 mg/kg and 1.0 mg/kg doses declined to baseline levels at 12 hours and then started to increase again after 12 hours (Figure 9). The 2.0 mg/kg dose declined slightly at 9 hours but remained elevated until 24 hours. These results indicate a significant effect of both dose and time in MT I mRNA expression ($p<0.0001$). The data for sham controls indicate that either there is a slight diurnal cycle for MT I mRNA levels with levels peaking at 4 p.m. and/or that the sham treatment had a minor effect on MT I mRNA kinetics.

The time course data for MT II mRNA expression exhibited a different pattern than that observed for MT I. For all three doses, a peak in MT II mRNA expression was observed at 1 hour with 2.0 mg/kg peaking the highest. This early peak was followed by a rapid decrease in MT II mRNA expression for all three doses returning to baseline

Table 5. Average C_T Values for MT II and GAPDH

Dilution	ng Total RNA	MT II Average C_T	GAPDH Average C_T	Log ng Total RNA	ΔC_T (MTII - GAPDH)
1	500	16.8±0.2	14.6±0.1	2.7	2.2±0.3
2	250	18.7±0.4	15.2±0.1	2.4	3.5±0.5
4	125	19.0±0.4	16.0±0.2	2.1	3.1±0.5
8	62.5	20.1±0.5	16.7±0.3	1.8	3.4±0.6
16	31.25	21.9±2.5	17.4±1.2	1.5	4.5±0.6
32	15.63	21.8±0.4	18.8±0.6	1.2	3.1±0.7
64	7.81	22.6±0.4	19.8±0.4	0.9	2.8±0.6
128	3.91	23.2±0.7	20.3±1.0	0.6	2.9±1.3
Slope	-0.0981				
Abs Val <0.1	0.0981				

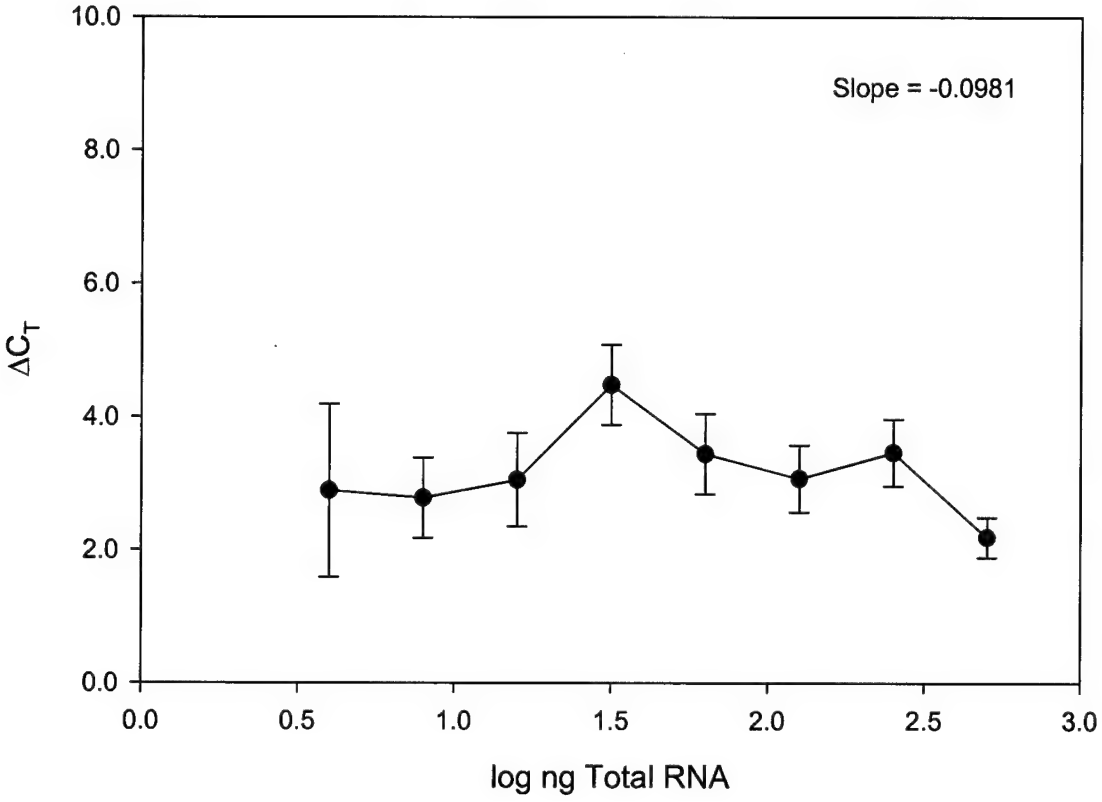

Figure 8. Relative efficiency plot of MT II and GAPDH.

levels at 3 hours (Figure 10). At 9 hours all three doses peaked again. Both the 0.5 mg/kg and 1.0 mg/kg doses declined at 12 hours and then increased again at 24 hours (Figure 10). The 2.0 mg/kg remained elevated out to 24 hours. From these results it is concluded that MT II mRNA expression is significantly effected by both time and dose also ($p<0.0001$).

To investigate the hypothesis that expression of MT I and MT II mRNA are coordinately regulated, the time courses of MT I and MT II mRNA expression were compared at each dose level. At the 0.5 mg/kg dose, MT II shows an initial peak at 1 hour followed by a decline at 3-6 hours (Figure 11). As MT II decreases, MT I increases at 3-6 hours and then decreases to baseline levels at 12 hours (Figure 11). MT II then peaks again at 9 hours. Both MT I and MT II decrease to baseline levels at 12 hours and start to increase again after 12 hours (Figure11). Note that the levels of MT I expression are significantly higher than MT II throughout the time course. The 1.0 mg/kg dose exhibits a pattern of mRNA expression that is similar to the 0.5 mg/kg dose. MT II displays a peak at 1 hour followed by a decrease at 3-6 hours (Figure 12). During the decline of MT II mRNA expression, MT I peaks at 6 hours and then decreases to baseline levels by 12 hours, whereas MT II peaks again at 9 hours followed by a decrease to near baseline level at 18 hours (Figure 12). Both MT I and MT II increase again at 24 hours (Figure 12).

At the highest dose (2.0 mg/kg) both MT I and MT II mRNA expression follow similar patterns to that observed at the 0.5 mg/kg and 1.0 mg/kg doses up to 9 hours (Figure 13). MT II peaks at 1 hour followed by a decrease at 3-6 hours (Figure 13). MT

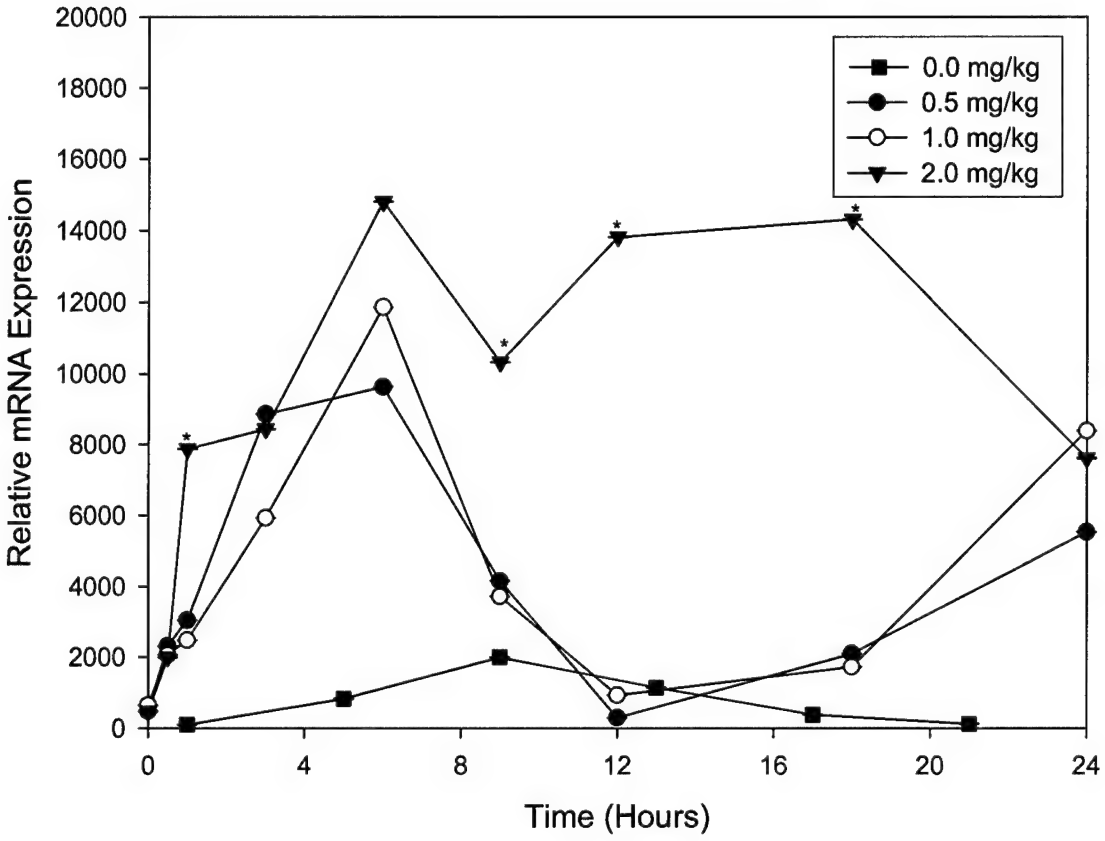

Figure 9. Comparison of 0.0, 0.5, 1.0 and 2.0 mg/kg Cd acetate on MT I mRNA expression. * Indicates significant difference between the means of the 0.5, 1.0 and 2.0 mg/kg doses at a specified time ($p=0.0001$, $p=0.0228$, $p<0.0001$ and $p=0.0109$). Note: the error bars were less than the symbol.

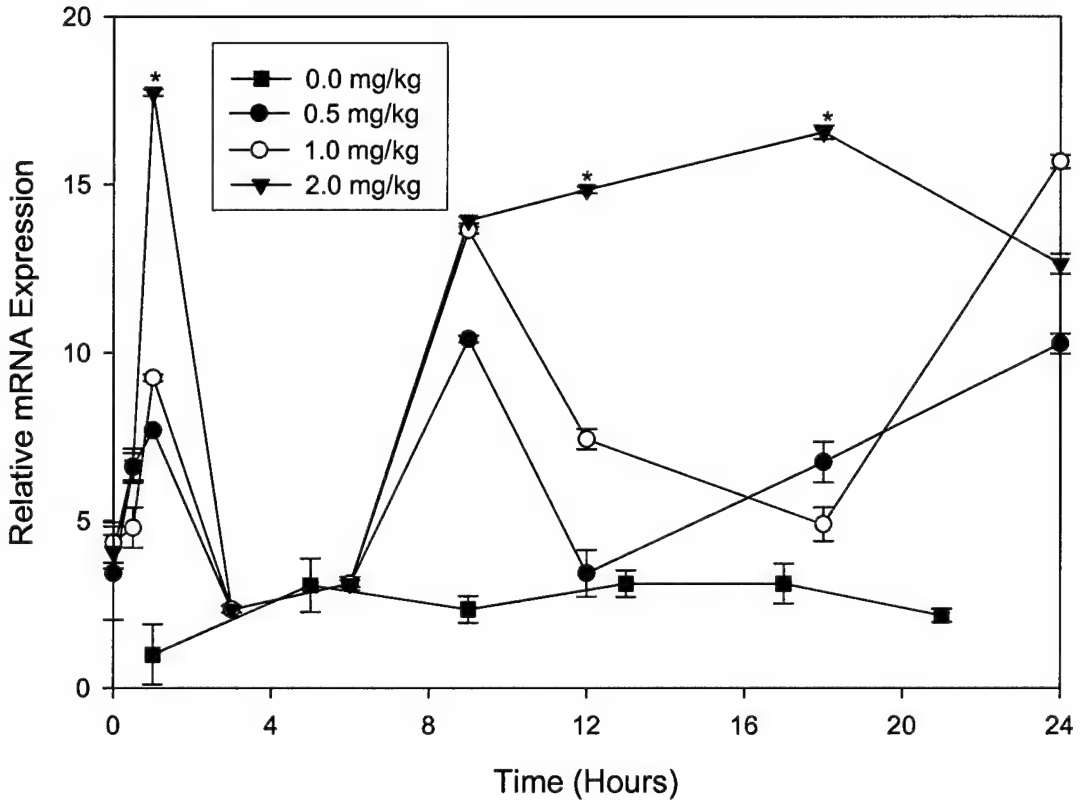

Figure 10. Comparison of 0.0, 0.5, 1.0 and 2.0 mg/kg Cd acetate on MT II mRNA expression. * Indicates significant difference between the means of the 0.5, 1.0 and 2.0 mg/kg doses at a specified time ($p=0.0001$, $p<0.0001$ and $p=0.0109$). Note: some error bars were less than the symbol.

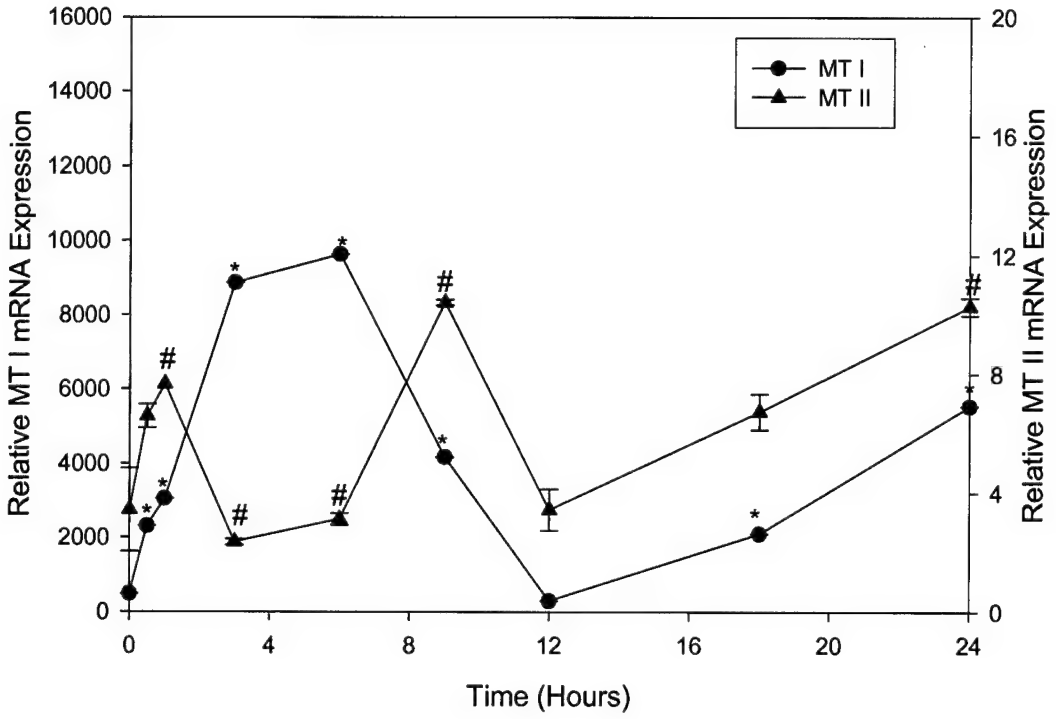

Figure 11. Comparison of MT I and MT II mRNA expression after administration of 0.5 mg/kg Cd acetate. *Indicates significant difference between MT I at various time points as compared to the 0 hour ($p<0.0001$). #Indicates significant difference between MT II at various time points as compared to the 0 hour ($p<0.0001$). Note: some error bars are smaller than the symbol.

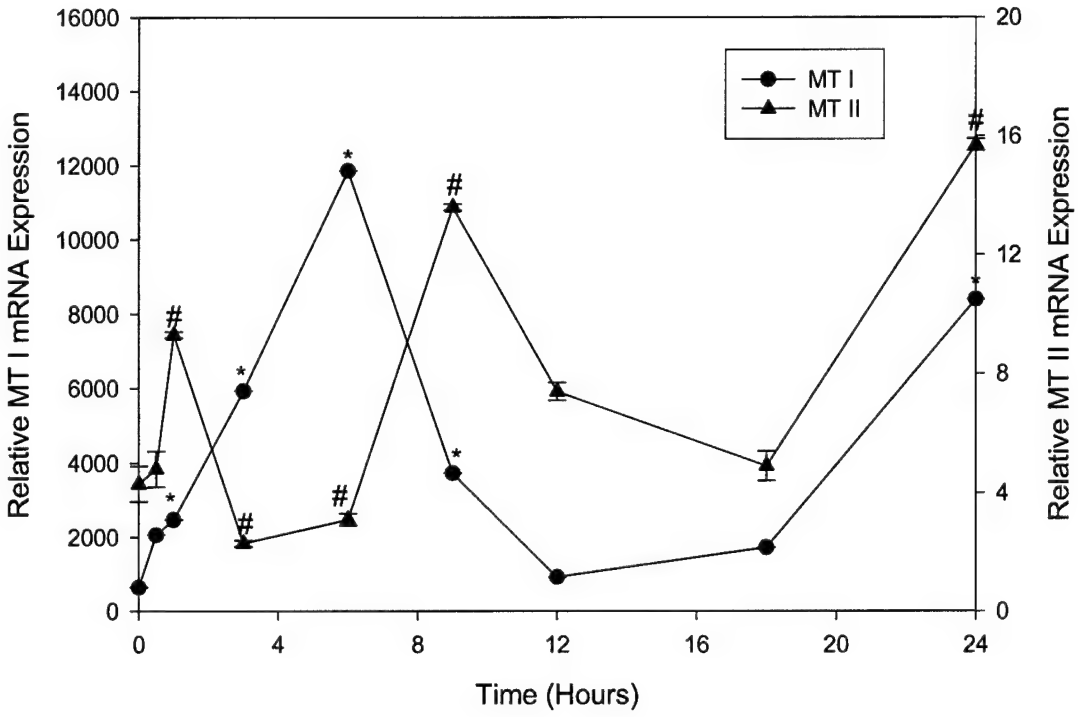

Figure 12. Comparison of MT I and MT II mRNA expression after administration of 1.0 mg/kg Cd acetate. *Indicates significant difference between MT I at various time points as compared to the 0 hour ($p<0.0001$). #Indicates significant difference between MT II at various points as compared to the 0 hour ($p<0.0001$). Note: some error bars are smaller than the symbol.

I peaks at 6 hours followed by slight decrease at 9 hours (Figure 13). MT II peaks for a second time at 9 hours. Both MT I and MT II remain elevated after 9 hours.

By comparing the time courses of both MT I and MT II mRNA expression, the coordination of the regulation of the expression of the two isoforms can be evaluated. The data strongly suggest that at early time points up to 12 hours after Cd injection, the expression of the two isoforms is not coordinated. At later time points, after 12 hours, the two isoforms show similar patterns of expression although the levels of mRNA expression are quite different.

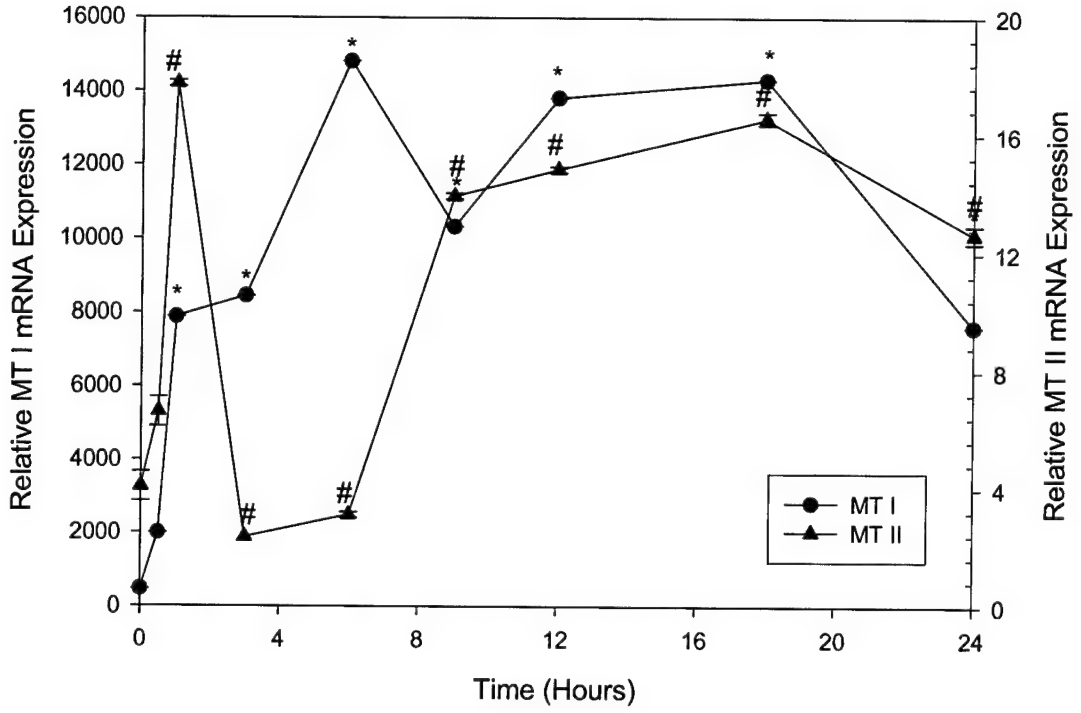

Figure 13. Comparison of MT I and MT II mRNA expression after administration of 2.0 mg/kg Cd acetate. *Indicates significant difference between MT I at various time points as compared to the 0 hour ($p<0.0001$). #Indicates significant difference between MT II at various points as compared to the 0 hour ($p<0.0001$). Note: the error bars are smaller than the symbol.

DISCUSSION

The fact that MT mRNA expression is induced by heavy metals is not a mystery. However, how the two isoforms (MT I and MT II) respond to heavy metals is still not clearly understood. It was the goal of this study to determine if the two isoforms are coordinately regulated in vivo. Using Real-Time PCR, it was possible to observe the expression of both MT I and MT II mRNA and evaluate their differences and similarities.

Both MT I and MT II mRNA expression are induced after administration of Cd. It was shown that both time and dose significantly affected the mRNA expression of both the isoforms. Even though both MT I and MT II showed a time and dose effect, their patterns of expression were very different. MT I showed a biphasic pattern at both the 0.5 mg/kg and 1.0 mg/kg doses. There was an initial peak at 6 hours followed by a return to baseline at 12 hours and a slight increase at 24 hours. This biphasic response has been reported in other studies of MT I mRNA expression (Vascondelos et. al 1996). It has been suggested that the second phase is caused by an inflammatory reaction to the toxic effects of the Cd injection (Vascondelos et. al 1996). However the results of this study were inconclusive. The mechanistic basis of the second induction phase of MT I still remains unclear. At the highest dose in the present study, 2.0 mg/kg dose, the time course of MT I mRNA induction did not exhibit this same biphasic response but instead peaked initially at 6 hours and remained elevated until 24 hours. It can be postulated that at the 2.0 mg/kg dose, a serious inflammatory response had occurred due to the toxicity

of Cd and that this could explain why the level of mRNA expression did not return to baseline as it did at the lower doses.

In contrast to MT I, MT II mRNA levels exhibited a triphasic response at both the 0.5 mg/kg and 1.0 mg/kg doses. In the literature, MT II was shown to exhibit a biphasic response similar to MT I but this conclusion is not contradictory as the earliest time point measured was 6 hours (Vascondelos et. al 1996). MT II showed a rapid response at 1 hour followed by a decline to baseline, another peak at 9 hours followed by another decline and finally a slight increase at 24 hours. As was observed with MT I mRNA levels, the 2.0 mg/kg dose did not exhibit the same response as the lower doses; following the second peak at 9 hours, MT II remained elevated until 24 hours. In fact at the 2.0 mg/kg dose, MT II displayed a more biphasic response.

In evaluating the responses of MT I and MT II at each dose level, it was evident that prior to 12 hours both MT I and MT II displayed very different patterns of mRNA expression. The effects were particularly prominent at the two lower doses where toxicity played a minor role. MT II peaked twice at 1 and 9 hours while MT I peaked only once at 6 hours. One possible explanation is that the isoforms exhibit different rates of mRNA synthesis and/or degradation. MT I exhibited a relatively slower rate of synthesis, taking MT I about 6 hours to reach its peak and another 6 hours to return to baseline levels. MT II exhibited a more rapid induction followed by a rapid decline. MT II peaked after 1 hour, returned to baseline at 3 hours followed by another rapid induction at 9 hours and a return to baseline at 12 hours. Thus, MT II mRNA levels appeared to respond more rapidly than MT I. MT II displayed a degradation of t ½ less than 1.5 hours. Although it is often assumed that synthesis and degradation of mRNA are

independent, it is possible that whatever factor is responsible for upregulation of the MT II gene is also responsible for downregulation of the MT I gene.

Prior to treatment with Cd, Zn is mostly bound to cellular protein resulting in a low level of free intracellular Zn. Under these conditions, MTF-1 is predominantly complexed with the inhibitor and the state of the cell is shifted to the left (Panel A, Figure 14).

Immediately after uptake of Cd into the cell, the reactions of the cell begin to shift to the right resulting in an increase in transcription. Cd displaces Zn that is bound to cellular protein increasing the amount of free intracellular Zn. As the intracellular concentration of Zn increases, it binds to the inhibitor which dissociates from MTF-1 allowing MTF-1 to bind to the MRE thus enhancing the initiation rate of transcription. Another driving reaction that is occurring at the same time is the complexing of Cd to cellular proteins which decreases the cellular protein binding sites available for Zn binding contributing to the increase in the concentration of free Zn within the cell (Panel B, Figure 14).

As MT protein is produced, both Cd and Zn are bound to the newly synthesized MT and the pool of free Cd and Zn decreases. In addition, the decrease in free Cd allows Zn to re-bind to cellular proteins also decreasing the concentration of free Zn. This decrease of free Zn within the cell increases the available pool of inhibitor that then binds to MTF-1. Once the MTF-1-inhibitor complex is formed, the rate of transcription returns to basal levels. (Panel C, Figure 14).

Figure 14 depicts the overall reactions involved in the regulation of MT I and MT II mRNA expression but this does not account for the differences in their mRNA expression. It could be hypothesized that MT II requires a different, and as yet unidentified, MTF-1 than MT I, which results in MT II being more sensitive to the increase in free Zn and thus a faster mRNA response. On the other hand it could be theorized that both the isoforms contain the same MTF-1 but the local sequences modulate MRE reactivity to MTF-1 causing a difference in mRNA expression.

It should be noted that MT I mRNA was always present at a much greater quantity than MT II mRNA and MT I mRNA induction was also of a much greater magnitude on an absolute scale. Previous studies have shown that the induction of MT I mRNA was considerably greater than that of MT II (10-fold as opposed to 3-fold) (Vascondelos et. al 1996). One theory to support this is that MT I and MT II proteins may have different affinities for different metals and hence lead to a differential response to these metals at the mRNA level. In humans MT II differs from MT I in amino acid composition, and this difference in amino acid composition affects the metal binding affinity of the two isoforms. In humans, MT I is rich in Cd and MT II is rich in Zn (Nordberg and Nordberg, 2000). Other studies have shown a different pattern of MT mRNA expression when comparing Cu and Cd exposure (Vascondelos et. al 1996). Cu induced MT II mRNA more than MT I mRNA and exhibited a monophasic response which remained elevated up to 12 hours. Cd on the other hand induced MT I mRNA more than MT II mRNA exhibiting a biphasic response in which the first phase was over by 12 hours. This latter response is similar to the results obtained in these experiments. These observations support the theory that the isoforms exhibit metal preferences.

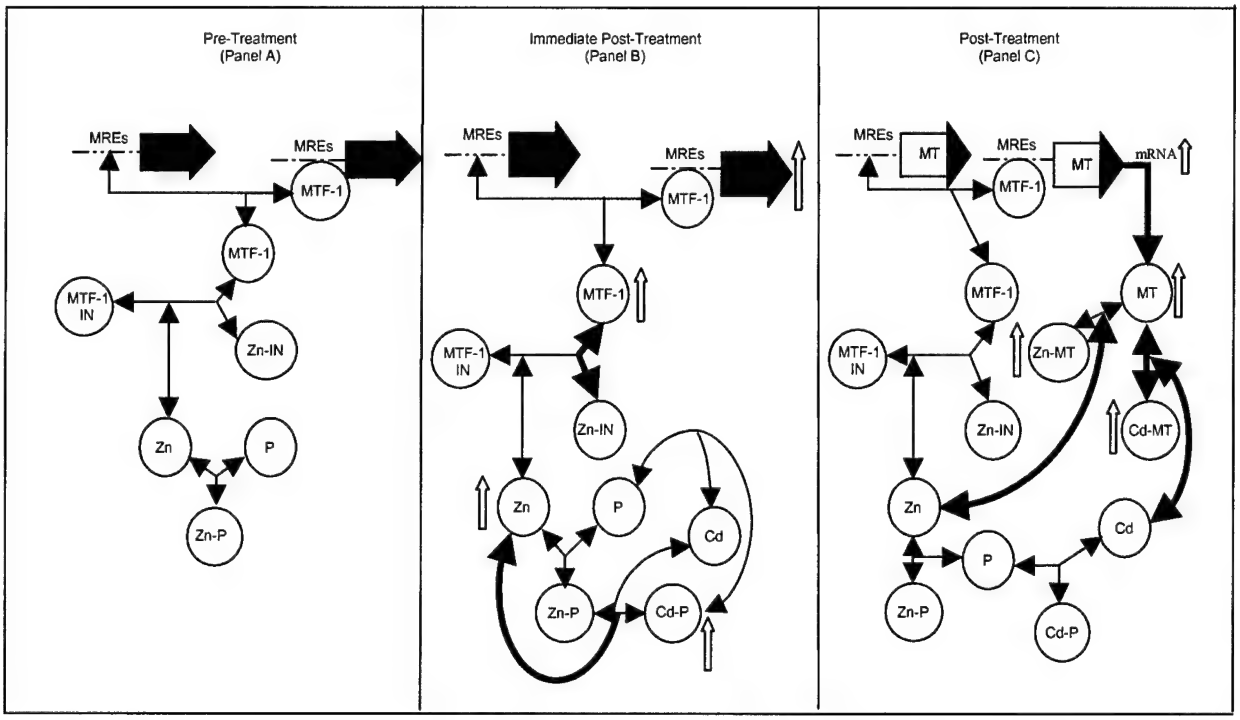

Figure 14. Proposed model of MT gene regulation by metals. Panel A depicts the proposed MT gene regulation prior to treatment with Cd. Under normal cellular conditions Zn is bound to cellular proteins and MTF-1 is bound to a Zn-sensitive inhibitor (IN). When free intracellular Zn concentrations are increased, Zn binds to IN allowing MTF-1 to bind to MREs on the MT gene and initiate transcription. Note: even under normal conditions minimal transcription is occurring.

In panel B, immediately post-treatment with Cd, the increase in free intracellular Zn occurs due to Cd displacing Zn from cellular proteins and Cd binding to cellular proteins. Since the concentration of free intracellular Zn increases, Zn binds to IN and allows MTF-1 to bind MREs on the MT gene causing a significant elevation in transcription.

Post treatment, panel C, exhibits an increase in MT protein which binds Cd and Zn allowing the free intracellular Zn levels in the cell to return to basal levels. During this phase, transcription is occurring at approximately the same rate as it is declining. Eventually the cell will return to its pre-treatment state with the exception of having Cd-MT present within the cell.

The observations found in this study do not support the hypothesis that the two isoforms of MT are coordinately regulated in vivo. Instead it supports the idea that the two isoforms play different roles in the detoxification of heavy metals and that different factors may play a role in their mRNA induction. Since MT I and MT II are encoded by different genes, there is some evidence that the expression of some of these genes is under separate control and may serve different biological purposes (Sadhu and Gedamu, 1988; Jahroudi et al 1990). This may help to explain the differences in both the isoforms mRNA pattern of expression.

Further studies would be needed to be performed to determine exactly what other factors beside metals may be involved in upregulation and downregulation of both the MT I and MT II gene following Cd exposure. It would also be beneficial to explore why there is a different initial induction response between the two isoforms but a similar response after 12 hours and how this relates to Cd hepatotoxicity.

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